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METHOD OF ANALYSIS

DETERMINATION OF WAX CONTENT BY CAPILLARY COLUMN GAS CHROMATOGRAPHY

1. SCOPE

This method enables the determination of the wax content of olive oils. The individual waxes are separated according to the number of carbon atoms. The method is recommended for distinguishing between olive oil obtained by pressing and that obtained from olive pomace (olive-pomace oil).

2. PRINCIPLE

Addition of a suitable internal standard to the oil, then fractionation by chromatography on a hydrated silica gel column. Recovery of the fraction eluted under the test conditions (which has a lower polarity than the triglycerides), then direct analysis by capillary column gas chromatography.

3. APPARATUS

3.1. Erlenmeyer flask, 25-ml.

3.2. Glass column for liquid chromatography, internal diameter 15.0 mm, length 30-40 cm, fitted with a suitable stop-cock.

3.3. Gas chromatograph for use with a capillary column, equipped with a system for direct on-column injection comprising:

3.3.1. Thermostat-controlled oven with temperature programming.

3.3.2. Cold injector for on-column injection.

3.3.3. Flame-ionisation detector and **converter-amplifier**.

3.3.4. Recorder-integrator (*Note 1*) for use with the converter-amplifier (3.3.3.), rate of response below 1 second, with variable paper speed.

3.3.5. Capillary column, glass or **fused silica**, 8-12 m length, 0.25-0.32 mm internal diameter, internally coated with liquid phase (*Note 2*) to a uniform thickness of 0.10-0.30 µm.

3.4. Microsyringe for on-column injection, 10 µ1, with a hardened needle.

3.5. Electric shaker.

3.6. Rotary evaporator.

3.7. Muffle oven.

3.8. Analytical balance for weighing to an accuracy of within ± 0.1 mg.

3.9. Usual laboratory glassware.

4. REAGENTS

4.1 Silica gel, 60-200 µm mesh. Place the gel in the muffle oven at 500 ºC for at least 4 hours. Allow to cool, then add 2% water in relation to the quantity of silica gel used. Shake well to homogenise slurry. Keep in darkness for at least 12 hours prior to use.

4.2. n-hexane, for chromatography.

WARNING - Fumes may ignite. Keep away from sources of heat, sparks, or naked flames. Make sure the bottles are always properly closed. Ensure proper ventilation during usage. Avoid build-up of fumes and remove any possible fire risk, such as heaters or electric apparatus not manufactured from non-inflammable material. Pernicious if inhaled since it can damage the cells of the nervous system. Avoid breathing in the fumes. Use a suitable respiratory apparatus if necessary. Avoid contact with eyes and skin.

Note 1. Computerised systems may also be used where the gas chromatographic data are entered through a PC.

Note 2. Suitable commercial liquid phases are available for this purpose such as SE52, SE54, etc.

4.3. **Ethyl ether**, for chromatography.

WARNING – Highly inflammable and moderately toxic. Skin irritant; pernicious if inhaled. May cause damage to eyes. Effects may be delayed. It can form explosive peroxides. Fumes may ignite. Keep away from sources of heat, sparks, or naked flames. Make sure the bottles are always properly closed. Ensure proper ventilation during usage. Avoid build-up of fumes and remove any possible fire risk, such as heaters or electric apparatus not manufactured from non-inflammable material. Do not evaporate to dryness or quasi-dryness. The addition of water or an appropriate reducing agent can reduce peroxide formation. Do not drink. Avoid breathing in the fumes. Avoid prolonged or repeated contact with skin.

4.4. **n-heptane,** for chromatography.

WARNING – Inflammable; pernicious if inhaled. Keep away from sources of heat, sparks, or naked flames. Make sure the bottles are always properly closed. Ensure proper ventilation during usage. Avoid prolonged breathing of fumes. Avoid prolonged or repeated contact with skin.

4.5. **Standard solution of lauryl arachidate** (*Note 3*), at 0.1% (m/v) in hexane (internal standard).

4.5.1. **Sudan I (1-phenylazo-2-naphthol).**

4.6. **Carrier gas:** hydrogen or helium, pure, for gas chromatography.

WARNING

Hydrogen. Highly inflammable, under pressure. Keep away from sources of heat, sparks, naked flames or electric apparatus not manufactured from non-inflammable material. Make sure the bottle valve is shut when not in use. Always use with a pressure reducer. Release the tension of the reducer spring before opening the bottle valve. Do not stand in front of the bottle outlet when opening the valve. Ensure proper ventilation during usage. Do not transfer hydrogen from one bottle to another. Do not mix gas in the bottle. Make sure the bottles cannot be knocked over. Keep them away from sunlight and sources of heat. Store in a corrosion-free environment. Do not use damaged or unlabelled bottles.

Helium. Compressed gas at high pressure. It reduces the amount of oxygen available for breathing. Keep the bottle shut. Ensure proper ventilation during usage. Do not enter storage areas unless they are properly ventilated. Always use a pressure reducer. Release the tension of the reducer spring before opening the bottle valve. Do not transfer gas from one bottle to another. Do not mix gas in the bottle. Make sure the bottles cannot be knocked over. Do not stand in front of the bottle outlet when opening the valve. Keep the bottles away from sunlight and sources of heat. Store in a corrosion-free environment. Do not inhale. Use solely for technical purposes.

Note 3: Palmityl palmitate or myristyl stearate may also be used.

4.7 Auxiliary gases:

- Hydrogen, pure, for gas chromatography.
- Air, pure, for gas chromatography.

WARNING

Air. Compressed gas at high pressure. Use with caution in the presence of combustible substances as the self-ignition temperature of most of the organic compounds in the air is considerably lower under high pressure. Make sure the bottle valve is always closed when not in use. Always use a pressure reducer. Release the tension of the reducer spring before opening the bottle valve. Do not stand in front of the bottle outlet when opening the valve. Do not switch the gas from one bottle to another. Do not mix gas in the bottle. Make sure the bottles cannot be knocked over. Keep them away from sunlight or sources of heat. Store in a corrosion-free environment. Do not use damaged or unlabelled bottles. Air intended for technical purposes must not be used for inhaling or for respiratory apparatus

5. PROCEDURE

5.1. Preparation of the chromatographic column

Suspend 15 g of silica gel (4.1) in n-hexane (4.2) and introduce into the column (3.2). Allow to settle spontaneously. Complete settling with the aid of an electric shaker (3.5.) to make the chromatographic band more homogeneous. Percolate 30-ml n-hexane to remove any impurities. Weigh exactly about 500 mg of the sample into the 25-ml flask (3.1), using the analytical balance (3.8), and add a suitable amount of internal standard (4.5) depending on the assumed wax content, e.g. add 0.1 mg of lauryl arachidate in the case of olive oil, and 0.25-0.50 mg in the case of olive-pomace oil.

Transfer the prepared sample to the chromatographic column with the aid of two 2-ml portions of n-hexane (4.2).

Allow the solvent to flow to 1 mm above the upper level of the absorbent. Percolate a further 70 ml of n-hexane to remove any n-alkanes naturally present. Then start chromatographic elution by collecting 180 ml of the n-hexane/ethyl ether mixture (*Note 4*) (*Note 5*) at 99:1, at a flow of about 15 drops every 10 seconds. The environment in which sample elution takes place should be at ambient temperature.

Evaporate the resultant fraction in a rotary evaporator (3.6) until the solvent is almost removed. Remove the last 2 ml of solvent under a weak current of nitrogen, then add 2-4 ml n-heptane.

Note 4: The n-hexane/ethyl ether (99:1) mixture should be freshly prepared every day.

Note 5: 100 µl of Sudan I dye at 1% in the elution mixture can be added to the sample solution to check visually that the waxes are eluted properly.
The retention of the dye lies in between that of the waxes and triglycerides. Hence, when the dye reaches the bottom of the chromatographic column, elution has to be suspended because all the waxes have been eluted.

5.2. Gas chromatographic analysis

5.2.1. Preliminary procedure

Fit the column to the gas chromatograph (3.3), connecting the inlet port to the on-column system and the outlet port to the detector. Check the gas chromatography apparatus (operation of gas loops, detector and recorder efficiency, etc.).

If the column is being used for the first time, it is advisable to condition it. Run a light flow of gas through the column, then switch on the gas chromatography apparatus. Gradually heat until a temperature of 350 ^{o}C is reached after approximately 4 hours.

Maintain this temperature for at least 2 hours, then regulate the apparatus to the operating conditions (regulate gas flow, light flame, connect to electronic recorder (3.3.4), regulate oven temperature for column, regulate detector, etc.). Record the signal at a sensitivity at least twice as high as that required for the analysis. The base line should be linear, with no peaks of any kind, and must not have any drift.

Negative straight-line drift indicates that the column connections are not correct; positive drift indicates that the column has not been properly conditioned.

5.2.2. Choice of operating conditions (*Note 6*)

The operating conditions are generally as follows:

- Column temperature:

  80 °C at first (1') —20 °C/min→ 240 °C —5 °C/min→ 325 °C (6') —20 °C/min→ 340 °C (10')

- Detector temperature: 350 ºC.

- Amount injected: 1 µl of n-heptane solution (2-4 ml).

- Carrier gas: helium or hydrogen at the optimal linear speed for the gas chosen (see Appendix A).

- Instrument sensitivity: suitable for fulfilling the above conditions

These conditions may be modified to suit the characteristics of the column and the gas chromatograph in order to separate all the waxes and to obtain satisfactory peak separation (see figure). The retention time of the internal standard must be 18 $\pm$ 3 minutes and the most representative peak of the waxes must be over 60% of the full-scale value.

Determine the peak integration parameters in such a way as to obtain a correct evaluation of the peak areas considered.

Note 6: Due to the high final temperature, positive drift is allowed but may not exceed more than 10% of the full-scale value .

5.3. Performance of the analysis

Take up 1 µl of the solution with the aid of the 10 µl micro-syringe; draw back the plunger until the needle is empty. Introduce the needle in the injection system and inject quickly after 1-2 seconds. After about 5 seconds, gently extract the needle.

Perform the recording until the waxes are completely eluted.

The base line must always satisfy the required conditions.

5.4. Peak identification

Identify the peaks from the retention times by comparing them with mixtures of waxes with known retention times, analysed under the same conditions.

The figure provides a chromatogram of the waxes in a virgin olive oil.

5.5. Quantitative analysis

Determine the areas of the peaks corresponding to the internal standard and the aliphatic esters from C40 to C46 with the aid of the integrator.

Determine the wax content of each ester, in mg/kg of fat, according to the formula:

$$\text{Ester, mg/kg} = \frac{A_x \cdot m_s \cdot 1000}{A_s \cdot m}$$

where:

- A_x = area corresponding to the peak for each single ester in square millimetres;
- A_s = area corresponding to the internal standard peak, in square millimetres;
- m_s = mass of the internal standard added, in milligrams;
- m = mass of the sample taken for determination, in grams.

6. EXPRESSION OF THE RESULTS

Report the sum of the contents of the different waxes from C40 to C46 (*Note 7*) in mg/kg of fat (ppm).

Results should be expressed to one decimal place.

Note 7: The components for quantification refer to the peaks with even carbon numbers amongst the C40-C46 esters, according to the specimen chromatogram of the waxes in olive oil provided in the attached figure. For identification purposes, if the C46 ester is split, it is recommended to analyse the wax fraction of an olive-pomace oil where the C46 peak is distinguishable because it is clearly predominant.

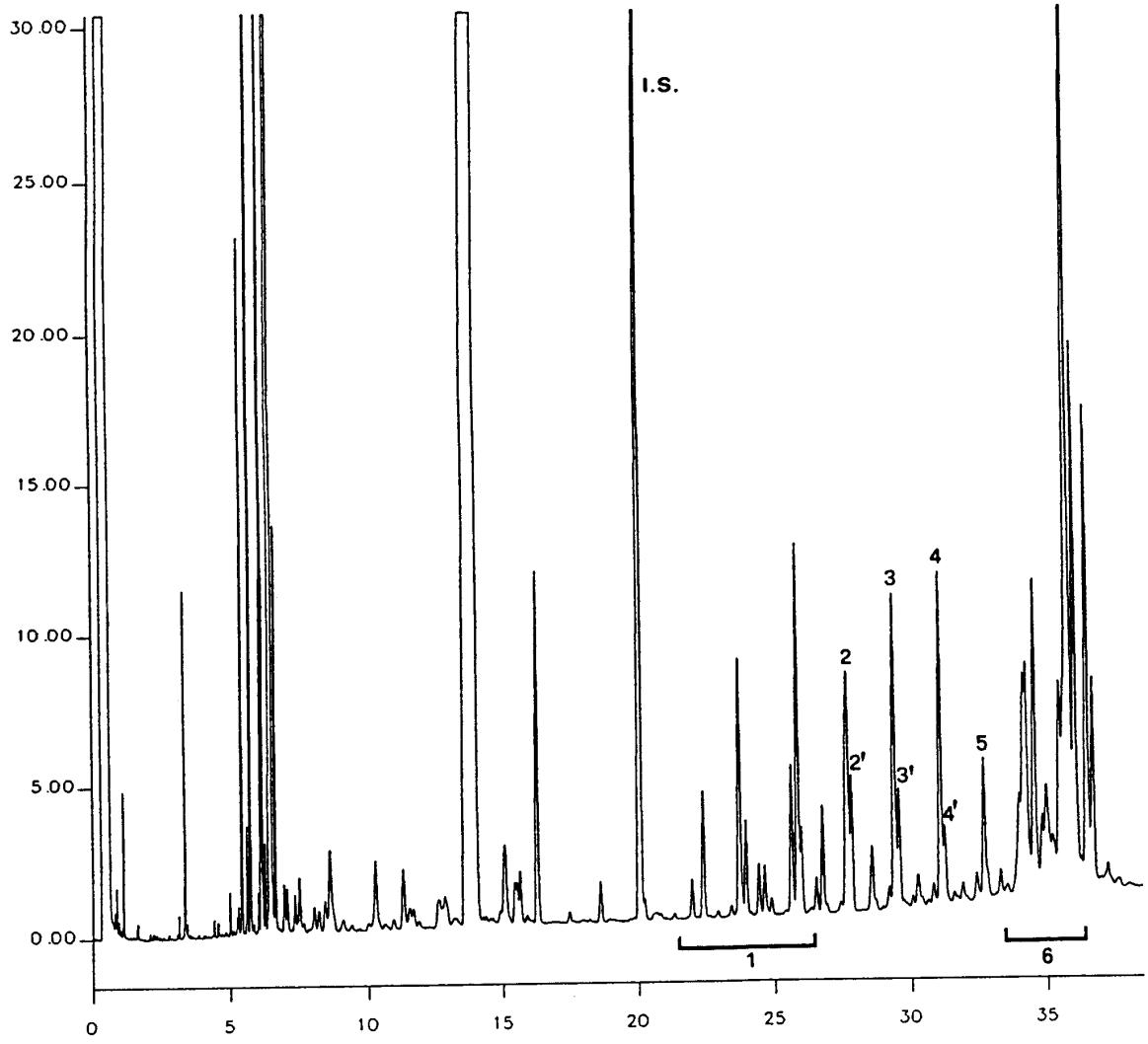

FIGURE: Specimen of a gas chromatogram of the wax fraction of an olive oil (*)

I.S. = Lauryl arachidate

1 = Diterpenic esters
2+2' = C40 esters
3+3' = C42 esters
4+4' = C44 esters
5 = C46 esters
6 = Sterol esters and triterpene alcohols

(*) After elution of the sterol esters, the gas chromatograph trace should not show any significant peaks (triglycerides)

Appendix

Determination of linear gas speed

Inject 1-3 µl of methane (or propane) into the gas chromatographic apparatus after adjusting it to the normal operating conditions. Measure the time the gas takes to run through the column from the moment it is injected until the peak emerges (tM).

The linear speed in cm/sec is given by the formula L/tM where L is the length of the column, in cm, and tM is the time measured in seconds.

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PRECISION VALUES OF THE METHOD

1. Analysis of the collaborative test results

The precision values of the method are given in the table overleaf.

Nineteen laboratories holding IOOC recognition at the time took part in the collaborative test arranged by the Executive Secretariat in 1999. The laboratories were from eight countries.

The test was performed on five samples:

A: extra virgin olive oil
B: virgin olive oil + refined sunflower oil
C: virgin olive oil + refined olive-pomace oil
D: virgin olive oil + refined soybean oil + refined sunflower oil
E: refined olive oil + refined olive-pomace oil + refined soybean oil + lampante virgin olive oil

The results of the collaborative test organised by the IOOC Executive Secretariat have been statistically processed according to the rules laid down in the international standards ISO 5725 **Accuracy (trueness and precision) of measurement methods and results.** Outliers were examined by applying Cochran's and Grubbs' test to the laboratory results for each determination (replicates a and b) and each sample.

The table lists:

n number of participating laboratories

outliers number of laboratories with outlying values

mean mean of the accepted results

r value below which the absolute difference between two single independent test results obtained with the same method on identical test material in the same laboratory by the same operator using the same equipment within short intervals of time may be expected to lie with a probability of 95%

S_r Repeatability standard deviation

RDS_r (%) Repeatability coefficient of variation (S_r x 100/mean)

R value below which the absolute difference between two single test results obtained with the same method on identical test material in different laboratories with different operators using different equipment may be expected to lie with a probability of 95%

S_R Reproducibility standard deviation

RDS_R (%) Reproducibility coefficient of variation (S_R x 100/mean)

Wax content (mg/kg)

	A	B	C	D	E
n	19	19	19	19	19
outliers	5	5	4	3	5
mean	120.32	123.14	222.41	174.1	345.93
r	9.51	12.56	10.51	12.22	14.91
S_r	3.39	4.48	3.75	4.72	5.32
RSD_r (%)	2.82	3.64	1.69	2.71	1.54
R	38.83	48.89	58.93	25.65	44.39
S_R	13.86	17.46	21.04	9.16	15.85
RSD_R(%)	11.53	14.18	9.46	5.26	4.58

2. References

ISO 5725-1: 1994 Accuracy (trueness and precision) of measurement methods and results – Part 1: General principles and definitions

ISO 5725-2: 1994 Accuracy (trueness and precision) of measurement methods and results – Part 2: Basic method for the determination of the repeatability and reproducibility of a standard measurement method

ISO 5725-5: 1994 Accuracy (trueness and precision) of measurement methods and results – Part 5: Alternative methods for the determination of the precision of a standard measurement method

ISO 5725-6: 1994 Accuracy (trueness and precision) of measurement methods and results – Part 6: Use in practice of accuracy values

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